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FINAL TECHNICAL REPORT

1. Use of KC-135 parabolic flights to determine if brief changes in the gravity field can influence the phase and/or period of the circadian clock

In February, 1994, a total of 50 hamsters flew on two separate KC-135 flights. On one flight (February 8th), 25 animals experienced 31 parabolas, thus going through 31 cycles of hypergravity (up to about 1.8 G) and microgravity (between 10^{-2} and 10^{-3}G). On the other flight, two days later, the animals were exposed to 43 parabolas. Fifty additional animals served as ground based controls and were treated in the same fashion as the experimental animals, except they were not loaded on board the aircraft, and during the flights they remained onboard the transport vehicle which drove through the streets near the airport.

A total of 116 animals (purchased at nine weeks of age from Charles River at Lakeview) were originally held under an LD 14:10 light dark cycle and were maintained in the animal quarters at the University of Houston Health Science Center. Ninety-six of these animals were housed in light-tight chambers in individual cages equipped with a running wheel cage (6 cages per chamber). The rhythm of running wheel activity was recorded via an on-line computer data collection system (Penev et al., 1994; Penev et al., 1993). After a 10-day acclimation period, the animals either remained on the original LD cycle or were shifted to an LD 14:10 light cycle in which the lights went on and off 6, 12 or 18 hrs later. Five weeks later, after all of the shifted animals had re-entrained to the new light dark cycles, all 96 animals were transferred to constant darkness for 10-12 days. On the two test days, 80 of the 96 animals with the most precise free-running rhythms of locomotor activity were loaded into one of two specially constructed flight modules that each held 20 small cages, (24cm x 14cm x 13cm). The cages allowed freedom of movement. The transfers were performed in constant darkness (with the aid of an infrared viewer), and while in the flight modules the animals remained in total darkness. The two modules were loaded onboard a transport vehicle and driven from the animal quarters to the Ellington Air Force Base. After the flight, all the animals were returned to the animal quarters, and again in total darkness were transferred back into their original cages and maintained in constant darkness for an additional two weeks. The free-running rhythm of locomotor activity continued to be recorded. The module that was loaded on board the aircraft was identical to the module that held the ground-based control animals.

The remaining 20 animals were maintained continuously on an LD 14:10 cycle. On the two flight days, 10 animals were transferred to cages on top of the two modules. Thus, these animals were exposed to ambient lighting throughout the time they were outside of the animal quarters. The period of time from when the animals were removed and returned to the animal quarters coincided with the light portion of the LD14:10 cycle they were exposed to in the animal quarters. These 20 animals had been outfitted with an indwelling intra-atrial cannula two days prior to the test days. Blood was collected from the ten animals that flew onboard the KC-135 aircraft at four time points: 1) prior to the loading of the animals for transport to the Air Force Base, 2) after the animals had experienced 20 parabolas, 3) Within 10 minutes after the animals had been loaded off of the aircraft, and 4) 24 hrs after the initial blood sampling. Blood was collected at the same time points from the 10 animals that were transported on top of the ground-control module. Plasma levels of the two adrenal stress hormones produced in hamsters, cortisol and corticosterone, were subsequently measured by radioimmunoassay. Both of these adrenal hormones were measured because it appears they are regulated independently; while acute stress induces an elevation in both corticosterone and cortisol, only cortisol levels are increased following chronic stress (Ottenweller et al., 1985).

The effects of the ground-based control procedure and the parabolic flights on the phase of the free-running rhythm of locomotor activity are shown in the left-hand panels of Fig. 1. No consistent phase shifts were observed in response to either procedure, and the vast majority of animals showed either no detectable phase shift, or only shifts on the order of a few minutes. We were able to test phase points throughout the circadian cycle since the animals were entrained to four different LD 14:10 light cycles prior to the transfer to constant darkness. In addition to not affecting the phase of the activity rhythm, neither the flight or the ground-based control procedure had any clear effect on the free-running period of the activity rhythm regardless of when the test procedure occurred during the circadian cycle (Fig. 1: right-hand panels).

Previous studies have demonstrated that an increase in locomotor activity and/or arousal of hamsters at certain circadian phases can induce pronounced (i.e. on the order of 1-3 hrs) and consistent phase-dependent shifts (both phase advances and phase delays) in the rhythm of locomotor activity (For review,

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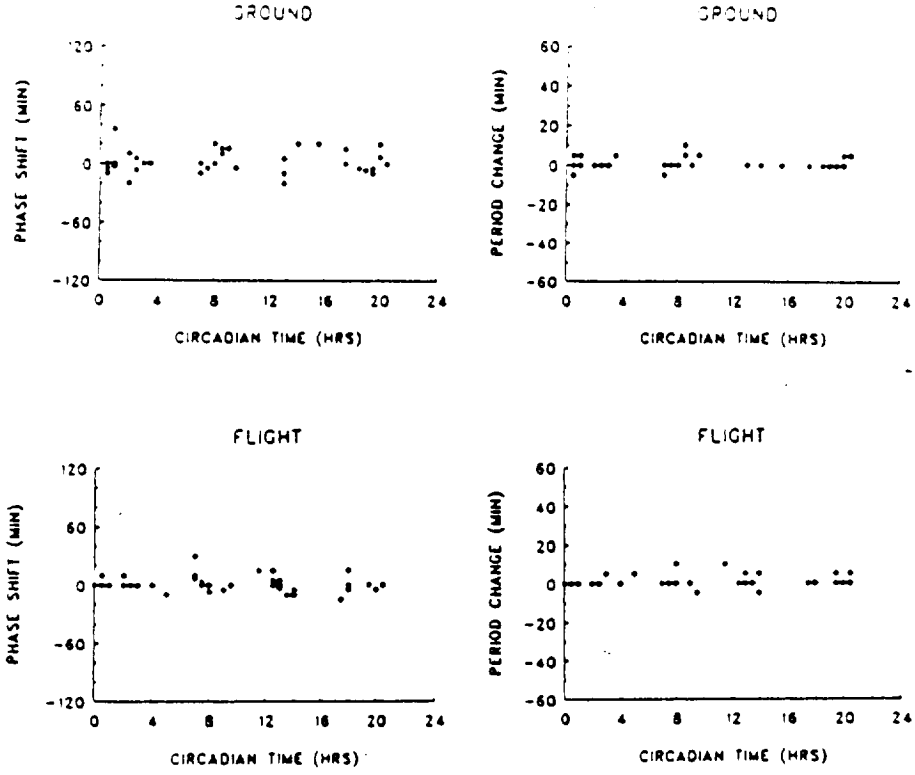
see (Turek, 1989)). Thus, there was some concern that the disturbances associated with loading and transporting the animals, and/or the loud noises associated with the flight itself, would induce "activity/arousal" type phase shifts in the hamsters. This clearly did not happen. Furthermore, while there is some indication that stress, as measured by an increase in adrenal hormone levels, might be able to phase shift the circadian clock at certain phases (Hastings et al., 1992), no phase shifts were observed despite the fact that there was a significant increase in both serum cortisol and corticosterone levels during and right after the flight when compared to blood levels prior to the flight (Fig. 2). Adrenal hormone levels had returned to the baseline levels by the time of blood sampling on the next day. No significant differences were observed in either cortisol or corticosterone levels at any of the time points in the ground-based control animals. Although we did not examine the effects of pulse changes in the gravity field on the pulsatile release of adrenal steroid hormones, the increased steroid levels were large enough to be significant without frequent blood sampling. Thus, exposure to the hypergravity/microgravity environment induced a significant increase in the adrenal stress hormones and had no effect on the circadian pacemaker of golden hamsters.

Behavioral observations were made on all of the control and flight animals that were not exposed to constant darkness. Since the cannulated hamsters were tested during the light phase, the animals were in their sleep phase throughout the period of observations. Indeed, following the exposure to microgravity, the animals were routinely observed to return immediately to the sleeping position. This was verified through the use of an onboard stationary video camera which photographed three of the animals on each flight during every parabolic maneuver. Thus, there were no obvious behavioral changes that indicated the animals were stressed, despite the clear increase in the adrenal stress hormones. Interestingly, squirrel monkeys have been observed to become drowsy and fall asleep after the onset of exposure to hypergravity (Fuller, 1984).

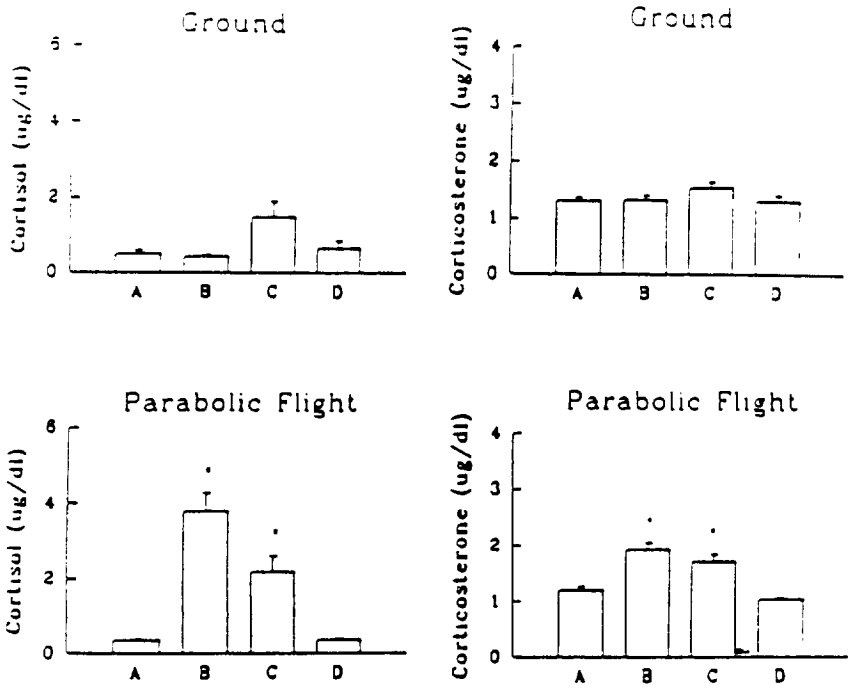
Three investigators (Turek, Penev and Van Reeth) flew with the animals during the first parabolic flight while two investigators (Turek and Penev) flew onboard the second flight. In addition to the new information on the effects of altered gravity states in hamsters, this experience has made us aware of what can and what cannot be done during the parabolic flights as well as of our own responses to the parabolic flights onboard the KC-135 aircraft. It also helped our team plan and perform the pilot studies associated with collecting blood from freely moving animals under conditions of hypergravity and microgravity described in section 2 of this report.

FIGURE 1



Changes in phase (left panels) and period (right panels) in both ground-based control animals (top panels) and in animals exposed to pulses of hypergravity/microgravity (bottom panels) during parabolic flight maneuvers. Both phase and period changes are plotted as a function of the onset of the control procedure or the parabolic flight maneuvers relative to the free running rhythm of activity. Circadian time 12 represents the onset of locomotor activity.

FIGURE 2



Circulating levels of the two adrenal stress hormones, cortisol (left panels) and corticosterone (right panels) in hamsters subject to the ground control procedure (top panels) or exposed to the hypergravity/microgravity pulses during parabolic flight maneuvers. A: levels on the morning of the test day, prior to loading the animals for transport to the airport. B: levels from animals bled on the ground at the same time as in animals bled after 20 parabolic flight maneuvers. C: levels from animals bled on the ground at the same time as in animals bled immediately after the parabolic flight. D: levels 24hrs after the initial blood sampling.

2. Effects of the parabolic flight on pulsatile hormone release: testing of blood sampling system in a hypogravity and hypergravity environment.

While we had flown on two previous KC-135 flights (see the first part of this section for description) when we had collected blood samples during 1G conditions, the April 25th flight was the first attempt to collect repeated blood samples in animals experiencing 1.8 G and zero-G conditions. Four investigators from Northwestern University were in Houston to perform these studies. The main objectives of this pilot study were: 1) to test whether freely moving animals can be bled during the 1.8 G and zero-G conditions of the parabolic flight; 2) to gain first-hand practical experience in order to improve our sampling techniques for future flights; and 3) to determine the optimal number of animals to be handled by each investigator under such circumstances.

a. Pre-Flight Activities

During the first week in April 1995, male golden hamsters (N=8) purchased at ten weeks of age from Charles River (New Jersey) were shipped directly to the University of Texas Health Science Center in Houston, Texas. The animals were maintained in individual polycarbonate cages (Nalgene, size C: 10" x 8" x 6") and held under an LD 14:10 light cycle with lights on from 0500 to 1900. Food and water were available ad libitum. Two days prior to the flight, the animals were outfitted with an indwelling intra-atrial cannula allowing the collection of serial venous blood samples (for details see Section E). On the afternoon before the flight day the animals were transferred into acrylic flight modules (4 hamsters/unit) with individual compartments of the same dimensions as the cages described above. On the day of the flight both modules with the animals arrived at the Ellington Air Force Base. A 0.1 cc sample of blood from all 8 animals was taken in order to get initial hematocrit readings. This procedure was repeated at the end of the serial sampling protocol to verify the maintenance of adequate blood substitution. Adapter lines were attached to the indwelling catheters of all animals in the flight and ground control groups at 06:00 am and the animals were left undisturbed until 7:00 am, when the continuous blood sampling protocol began. The four animals in the flight group were moved onboard the KC-135 aircraft at approximately 9:00 am. Pre-flight blood samples were collected from these as well as the four ground control animals at 15 minute intervals from 7AM until 9:15AM. The actual time of take-off was about 10:15 am.

b. In-Flight Activities

Two investigators (Turek and Penev) collected blood samples from the animals during the parabolic flight (Fig. 3). The first blood sample in-flight was taken during one of the first four parabolas at about 10:45 AM Blood sampling continued at approximately 15-min intervals throughout the flight from 3 of the 4 on-board animals. Due to displacement of the intra-atrial catheter we were unable to clear the blood collection line from the 4th animal (which occasionally happens during blood sampling under laboratory conditions as well) and thus we focused our attention and effort on the three remaining animals. All blood sampling procedures were recorded by an on-board stationary video camera, and each investigator provided a continuous audio record of their activities. This ensured the subsequent verification of the exact timing of all on-board blood collection procedures. Complete blood samples (0.5cc) from the animals were collected during 20-25 sec periods of both hypergravity and microgravity. Similarly, donor blood was successfully given back to the animals during both 1.8 G and zero-G conditions. Indeed, our subjective impression at this time is that it was actually easier to obtain a rapid blood sample during the Zero G conditions, when compared to collecting blood samples during 1.8 G or during 1.0 G ground conditions. Along with the in-flight blood collection, the serial sampling protocol of the four ground-based control animals resumed 30 minutes after KC-135 take-off and continued in a similar fashion at 15-minute intervals.

c. Post-Flight Activities

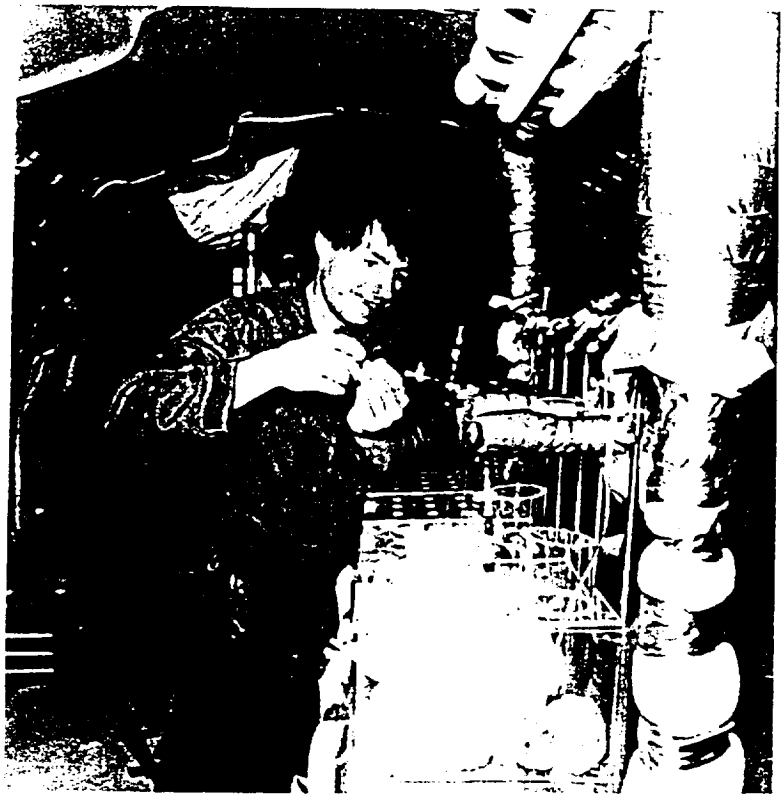
After landing, the serial sampling continued for another three hours in the three parabolic flight hamsters and the four ground control animals. All blood samples were centrifuged at the Ellington Air Force Base. Plasma was collected, frozen and shipped back to Northwestern University. Growth hormone (GH), cortisol and corticosterone levels were measured using a radioimmunoassay according to the methods described in section 3.

d. Summary and Conclusions

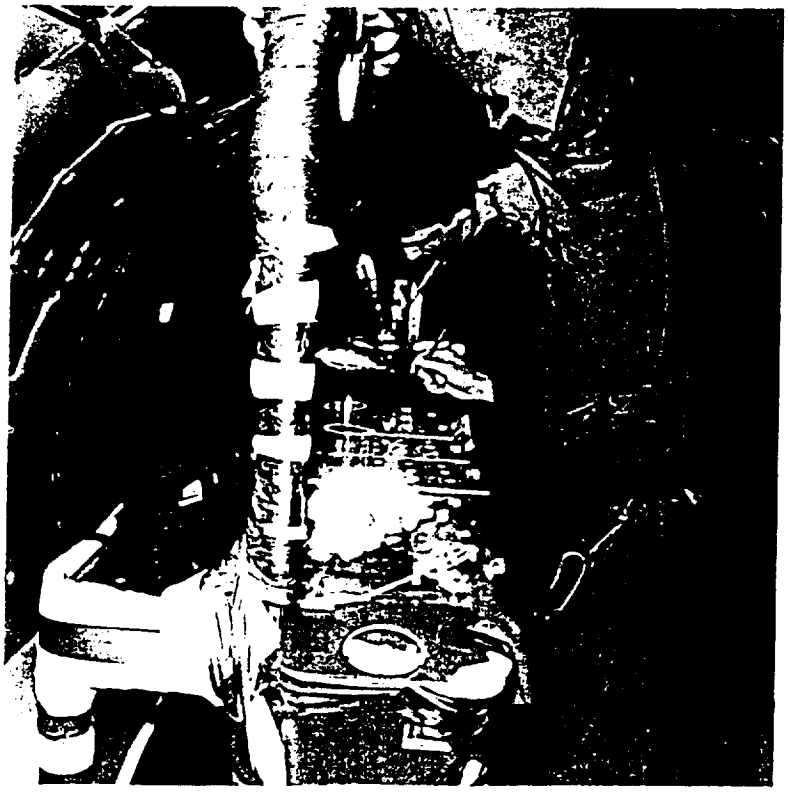
The profiles of plasma GH, cortisol and corticosterone from representative parabolic flight and ground-control animals during pre-flight, in-flight and post-flight conditions are shown in figure 4 and 5. We recognized that our small sample size would preclude us from making any firm conclusions about the effects of the flight on circulating hormone levels. Therefore, no statistical comparisons between or within the groups can be made with this small sample size. Nevertheless, our preliminary results indicate that pulsatile hormone release persists in the microgravity environment, and that adrenal steroid hormone levels are elevated in the flight, but not the ground control, animals. While all three parabolic flight animals showed elevated levels of cortisol and corticosterone both during and after the flight when compared to pre-flight levels, none of the four ground-based animals showed such comparable increases in circulating adrenal steroid levels. These data are consistent with our earlier finding that adrenal steroid levels are increased in response to the parabolic flight (see section 1 of this report and Fig. 2). Furthermore, the low levels of cortisol and corticosterone in the ground control animals is consistent with our previous studies demonstrating that the blood sampling procedure in itself does not result in elevated adrenal steroid levels.

The meaningful assessment of the effects of changes in the gravity field on hormonal secretion must take into account the pulsatile nature of hormonal release, and multiple samples must be taken from individual animals to accurately define the effects of an altered gravity environment on the endocrine system. Our pilot studies represent an initial and necessary first step for reaching this objective. We were successful in collecting repeated blood samples from animals in altered gravity environments. To our knowledge, this is the first demonstration that a serial blood sampling protocol can be used in conditions of microgravity. Most importantly, this valuable experience has enabled us to make several changes in the blood replacement and collection procedures (described in detail in section E) in order to improve the efficiency and reliability of our system under 1.8 G and zero-G conditions. Based on our flight experience we have developed a modified sampling protocol making it possible for a single investigator to collect blood from 4-5 experimental animals every 10 minutes during the parabolic flight.

Figure 3



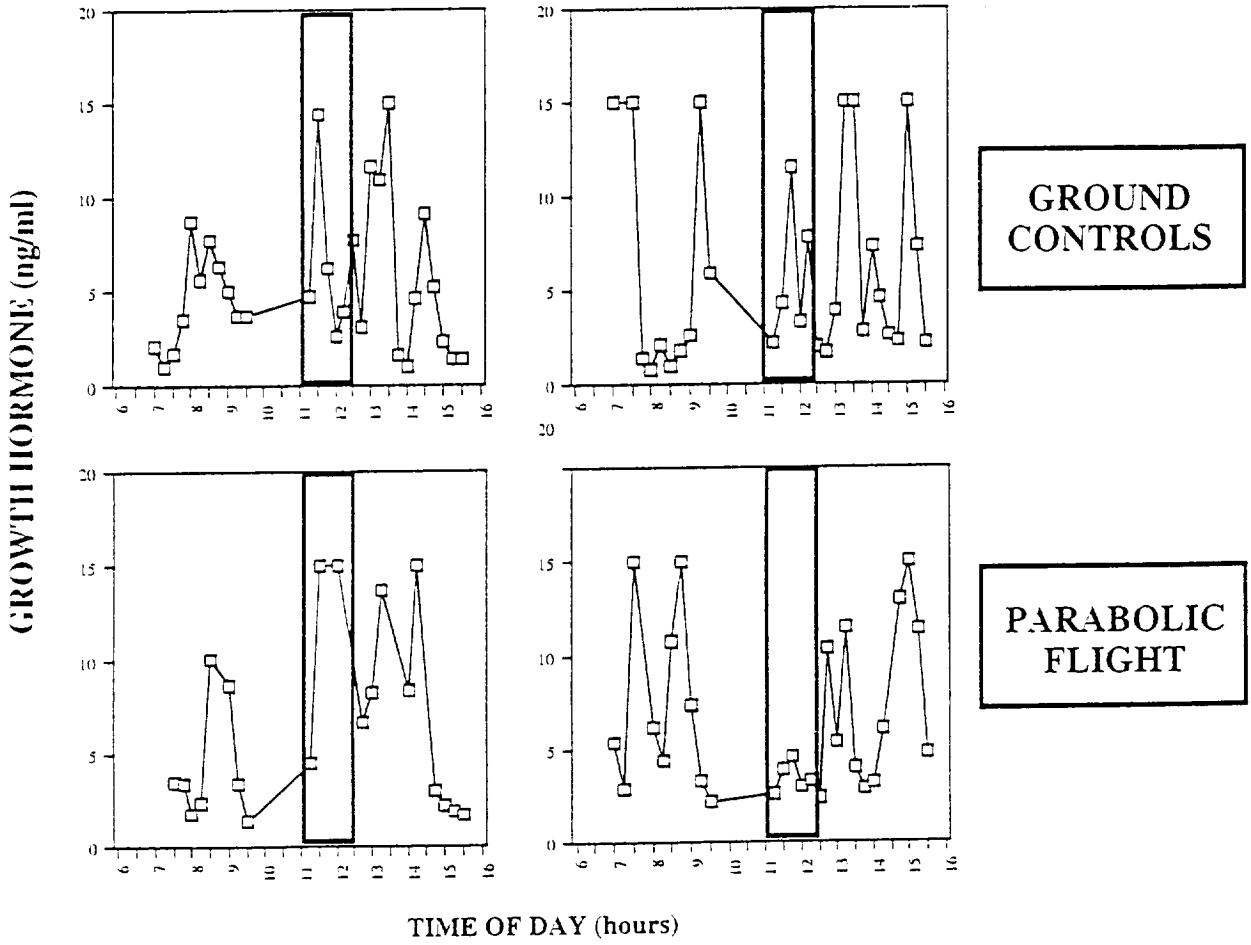
Resting hamster in 1-G



Floating hamster in Zero-G

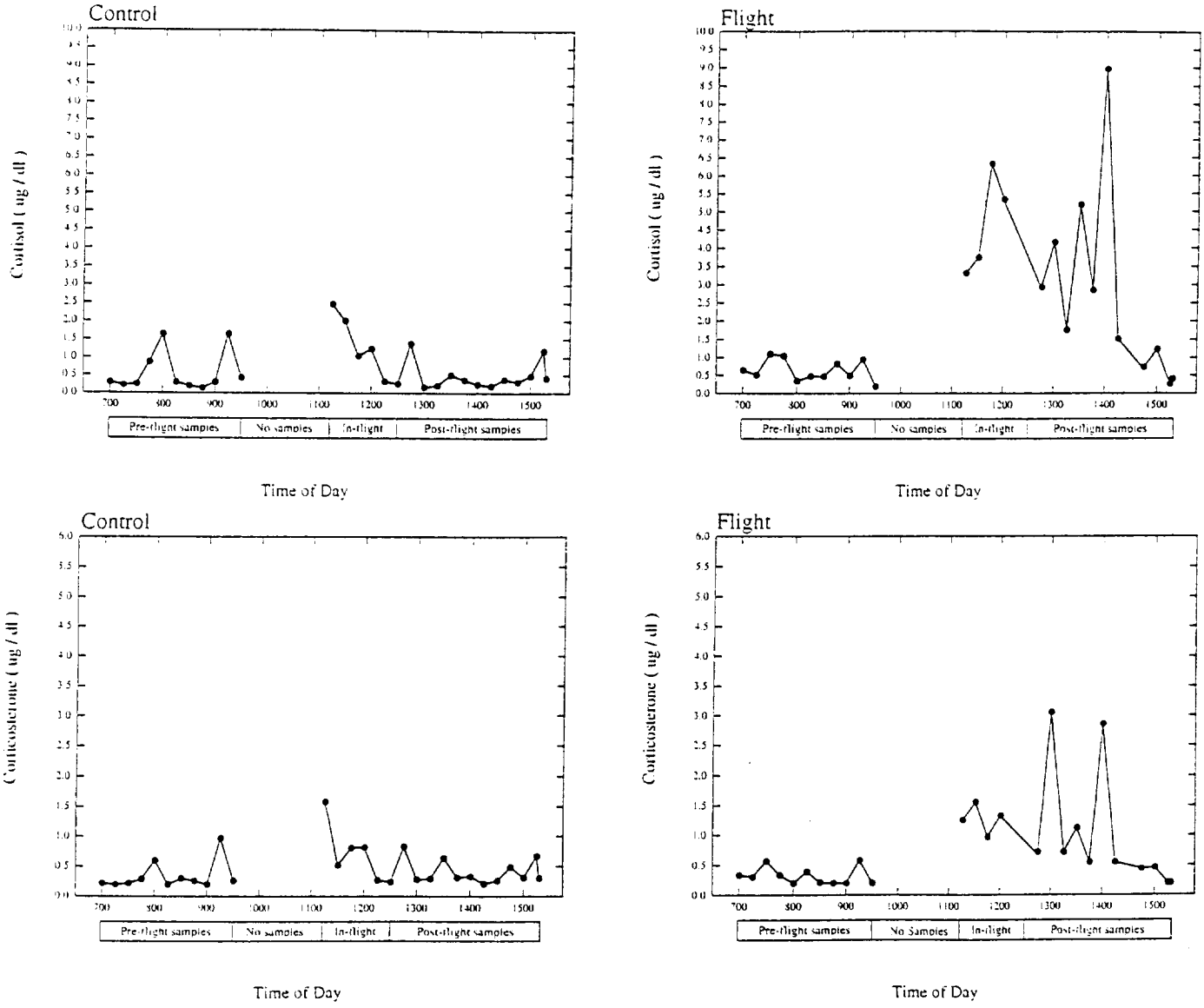
Two photographs showing the blood sampling system and investigators on board the KC-135 aircraft. In the upper photograph, the hamster is exposed to 1-G. The second photograph shows a hamster free-floating during a period of exposure to microgravity while a blood sample is being withdrawn.

Figure 4



Plasma growth hormone (GH) levels from two ground control and two parabolic flight animals. Samples were collected pre-flight, during the flight and for a three hour period post-flight. No blood was collected for about an hour before flight or during take-off and the period just prior to the first few parabolic maneuvers. The vertical rectangle denotes the period of time when the flight animals were exposed to the parabolas. Both of the ground control and one of the parabolic flight animals showed a clear pulsatile increase in plasma GH levels during the flight.

Figure 5



Plasma cortisol and corticosterone levels from a ground control hamster and a hamster experiencing the parabolic flight conditions. See Fig. 2 legend for further details. While plasma cortisol and corticosterone levels remained low throughout the blood sampling procedures in both of the control animals, both hormones were elevated during the flight and for at least the first two hours after the termination of the flight in the animal experiencing the hyper- and hypogravity conditions.

e. Description of Materials, Methods and Procedures

Surgery and Blood sampling

The successful completion of this project requires that animals be outfitted with an intra-caval/atrial dual catheter assembly developed in our laboratory (patent pending), that allows for frequent blood sampling and continuous automatic replacement with donor blood in freely moving animals. Below is a brief description of the procedures for collecting blood at frequent intervals (e.g., 10 minutes) for long periods of time (e.g., 24-48 hrs).

Cannula Construction: The dual catheter assembly is constructed of polyethylene and silastic tubing (Clay Adams/Intramedic and Dow Corning) and cut to a predetermined length (depending on the body weight of the animal) calculated for correct positioning of the sampling catheter at midpoint in the right atrium of the heart. Cannulas are stored in 20% ethanol and flushed with 3.0 cc of heparinized saline (5 unites heparin/ml) before surgery.

Surgery: Cannulas are implanted 2 days prior to blood sampling. Each animal is anesthetized with surital/ketamine HCl and injected with gentomycin sulfate (5.0 mg/kg) to prevent infection during surgery. An incision is made through the skin layer above the jugular vein. The salivary glands are carefully deflected to expose the right jugular vein. The vein is excised from its sheath with forceps and encircled with two lengths of suture. One suture is tied tightly, just caudal to the bifurcation of the vein to block the flow of blood from the head. The other suture is tied loosely just above the point where the vein enters the chest cavity. A 25 G needle, attached via tubing to an IV bag containing 0.9% sodium chloride, is inserted into the vein between the two sutures. The saline is allowed to flow through the needle to expand the vein for easier insertion of the cannula. The hole initiated by the needle is widened with forceps and the cannula is inserted. and tied with a suture. The free ends of the cannula are threaded under the skin toward the middle of the back and exit through an incision between the shoulder blades. The ends of the cannula are closed between experiments with a stainless steel pin. A plastic tube glued to the skin of the animal covers the external part of the cannula and prevents the hamster from damaging it. Following cannulation, the animals are housed individually and are allowed complete freedom of movement within their cages. Hamsters are observed to eat, drink and sleep without apparent discomfort within hours following this procedure.

Blood Sampling Procedure: An hour before the first sample is drawn, hematocrits are taken from each animal. Both catheters are flushed with heparinized saline (5.0 units / ml). A connector made of 23G stainless steel tubing approx. 1.0 cm in length is inserted into each catheter. A length of polyethylene tubing is attached to the connector/catheter for collecting blood samples. The end of the tubing is attached to a 23G hypodermic needle that is glued to a three way stopcock suspended over the cage. A 1.0cc syringe is attached to the upper port. Similarily, a length of tubing is attached to the other catheter via the s/s connector. It is then connected to a 23G swivel device which in turn is connected to a syringe filled with donor blood mounted in an infusion pump (KS Scientific). The pump is programed to deliver 50 ul/min of donor blood to the animal. The swivel device and the freely mounted stopcock will allow the animal to move in the cage without the catheters becoming tangled. Donor blood is heparinized (5.0 units per ml) so that the animal is receiving a constant ifusion of an anticoagulant as they receive the donor blood. This allows a constant draw of a blood sample at a specific time interval without having to clear the line with heparinized saline inbetween each sample collected. Blood samples are collected in a 1.0 cc syringe attached to the upper port of the stopcock. Once the sample is collected the syringe is removed and replaced with an empty 1.0 cc syringe, ready for the next sample. Hematocrits obtained before and after each experiment indicate that this procedure is sufficient to maintain each animal's blood cell count within normal levels (45-47%). All collected blood samples are kept on ice before centrifugation. Following centrifugation, the plasma is removed, snap frozen and stored at -70 deg. C until later determination of hormone levels by radioimmunoassay (see part 3 in this section).

Donor Blood Preparation: Whole hamster blood in citrate-phosphate-dextrose (CPD) solution is purchased from Charles River Laboratories. Blood is centrifuged (4°C; 2400 rpm; 5 min) and, after discarding the plasma, the cells are resuspended in saline and centrifuged a second time, rinsed with saline and centrifuged a third time. The final supernatant is discarded and the cells resuspended in a solution calculated to contain 45% hematocrit, 45% plasma proteins (Plasmanate; Cutter Laboratories), 10%

phosphate-dextrose. Heparin (5.0 units/ml) and gentomycin sulfate (0.1 mg/ml) are added to the donor blood which is then stored at 4°C and used within 72 hours. Aliquots of plasma from donor blood are examined in each assay to ensure the lack of detectable hormone levels in the donor blood.

Hormone Assays and Analysis

Hormone Assays: Prolactin, GH, TSH and LH will be assayed by radioimmunoassay utilizing reagents and instructions supplied by the NIDDK of the NIH for measuring pituitary hormones in the rat and all have been validated for the hamster. Recent papers describing the use of these NIH kits include, (Bruhn et al., 1992; Lafuente et al., 1993; Meredith et al., 1991). We have extensive experience with these kits and have been assaying peptides in the blood and pituitary glands of rodents for 20 years. ACTH will be assayed using the double antibody RIA kits for humans produced by ICN Biomedicals Inc. (Carson, California). Corticosterone and cortisol will be assayed using the double antibody RIA kit for rats and mice produced by ICN Biomedicals Inc. (Costa Mesa, California). We also have extensive experience in using RIA kits for steroid hormones. It should also be noted that Dr. Turek is Director of an NIH sponsored P-30 Center Grant on Fertility and Infertility (see Proposed Costs Section for information on this grant) that sponsors a radioimmunoassay core facility at Northwestern University. This core keeps the laboratories at Northwestern informed of the latest advances in hormone measurements, and is constantly developing and testing new procedures to improve the specificity and sensitivity of the assays.

Pulse Analysis: All hormones which we plan to study are secreted in a series of pulses. A computerized algorithm for pulse identification, called ULTRA, (Van Cauter, 1981; Van Cauter, 1988) will be used to quantify these rapid fluctuations. The general principle of this algorithm is the elimination of all peaks of plasma concentration for which either the increment (difference between the peak value and the preceding nadir) or the decrement (difference between the peak value and the next nadir) does not exceed a certain threshold. The increments and decrements are expressed as percent increase over the preceding nadir and percent decline from the peak, respectively, and compared to a multiple of the local intra-assay coefficient of variation in the relevant range of concentration. This local coefficient of variation (CV) is estimated, at every level of plasma concentration, by linearly interpolating between the values of the intra-assay coefficient of variation provided by the user for various concentration ranges. The peaks which do not meet the threshold criteria are eliminated from the series by an iterative process, leaving a series in which all local maxima represent significant pulses. This series is referred to as the "clean" series. Each pulse is then identified and characterized in terms of duration and magnitude of both the increment and the decrement.

Recent review articles (Evans et al., 1992; Royston, 1989; Urban et al., 1988) have provided comparisons of performance of several pulse detection algorithms including ULTRA (Van Cauter, 1981; Van Cauter, 1988), PULSAR (Merriam and Wachter, 1982), CYCLE DETECTOR (Clifton and Steiner, 1983), CLUSTER (Veldhuis and Johnson, 1986), and DETECT (Oerter et al., 1986). A comparative study of the performance of various pulse detection algorithms on experimental series conducted by Urban et al (Urban et al., 1988) indicated that ULTRA, CLUSTER and DETECT perform similarly when used with appropriate choices of parameters. Objective assessment of the performance of a pulse detection program may be by testing computer-generated profiles including both pulses and "noise" and examining false-positive and false-negative errors. This approach has been used by the authors of ULTRA, CLUSTER and DETECT (Genazzani and Rodbard, 1991; Guardabasso et al., 1988; Urban et al., 1989; Van Cauter, 1988), and these three algorithms provide similar results. We used the pulse detection and analysis algorithms in the ULTRA program for the analysis of hormonal profiles.

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